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Optical studies on the specific interaction of dipyridamole with α_1-acid glycoprotein (orosomucoid)

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The interaction of dipyridamole with α_1-acid glycoprotein was investigated by circular dichroism and ultraviolet absorbance measurements as well as by equilibrium dialysis experiments. Dipyridamole is bound to the protein via one site of extremely high affinity and by at least one site of considerably lower affinity. Only the association of dipyridamole with the high affinity site produces typical extrinsic Cotton effects. As a result of experimental observations it is concluded that the high affinity site is located in a hydrophobic protein structure of the glycoprotein.

α_1-Acid glycoprotein (α_1-AGP) is one of the glycoproteins found in mammalian blood and represents one of the biochemically best characterized glycoproteins. Its level in human serum varies widely in healthy subjects and is markedly elevated in a variety of unrelated diseases. During the last decade, α_1-AGP has attracted the attention of pharmacologists since it could be demonstrated that it represents the major binding component in human and animal serum for several basic drugs, including β-blockers, tricyclic antidepressants, and neuroleptics (Piafsky 1980). However, only few observations are available about the molecular aspects of the interaction of drugs with α_1-AGP (number of sites, affinity, location and structure of binding sites), preferentially made with pure α_1-AGP which contrast sharply with the information we have now about similar aspects of the interaction of drugs with serum albumins (Müller & Wollert 1979; Fehske et al 1981).

Possibly, the first demonstration of a strong binding of a basic drug to pure α_1-AGP was reported in 1971 for the vasoactive compound dipyridamole which is now used clinically as a antiplatelet and antithrombotic agent (Kopitar & Weisenberger 1971). Subsequent investigations showed that α_1-AGP strongly inhibits the dipyridamole-induced reduction of platelet aggregation possibly by preventing the binding of dipyridamole to platelets (Niewiarowski et al 1975; Subbarao et al 1977). Thus, the effectiveness of dipyridamole in patients may depend on the plasma concentration ratio between dipyridamole and α_1-AGP (Subbarao et al 1977).

The present publication reports some mainly optical experiments carried out to characterize the interaction of dipyridamole with α_1-AGP.

MATERIALS AND METHODS

Materials

α_1-acid glycoprotein (human) (α_1-AGP, electrophoretic purity 99%) and human serum albumin (HSA, electrophoretic purity 100%) were obtained from Behringwerke, Marburg (FRG). Dipyridamole and [^{14}C]dipyridamole (spec. act. 4 mCi mmol^{-1}) were gifts by Dr Karl Thomae Co., Biberach (FRG). All other chemicals were obtained from commercial suppliers and were of reagent grade. All solutions were prepared with deionized water.

Circular dichroism measurements

Circular dichroism (CD) measurements were carried out with a Cary 61 CD spectropolarimeter calibrated with (+)-camphorsulfonic acid. All spectra were recorded in cylindrical cells with 10 mm path length using a full scale deflection of 0·02° and spectral band width of 2 nm. All measurements were made in 0·05 M phosphate buffer pH 7·4. Results are expressed as molar ellipticity ([θ]) calculated with reference to the α_1-AGP concentration (25 μM).

Ultraviolet difference spectroscopy

Ultraviolet (u.v.) absorbance difference measurements were made with a spectrophotometer using

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tandem double compartment cells. α_1-AGP concentration was always 25 μM.

Equilibrium dialysis

Binding of [^{14}C]dipyridamole to α_1-AGP was determined by equilibrium dialysis using an α_1-AGP concentration of 25 μM and varying concentrations of the drug. All solutions were prepared with 0·05 M phosphate buffer pH 7·4. 0·9 ml of the protein solution was dialysed for 16 h at 25 °C in the dark against 0·9 ml of the buffer containing [^{14}C]dipyridamole. One ml dialysis cells and cellophan dialysis membranes (Union carbide) were used. The radioactivity at both sides was determined by liquid scintillation spectrometry.

Tyrosine and tryptophan modification

Tyrosine and tryptophan residues of α_1-AGP were modified as described for human serum albumin elsewhere (Fehske et al 1978, 1979). For the tyrosine residue modification, α_1-AGP was treated with a 5- or 20-fold molar excess of tetranitromethane in 50 mM Tris buffer at pH 8·0 resulting in derivatives with a degree of modification of 1·2 or 4·4 respectively as determined spectrophotometrically (Fehske et al 1979). The tryptophan modification was performed with 2-hydroxy-5-nitrobenzyl bromide at a 40-fold molar excess in 10 M urea at pH 4·1 giving a derivative with a degree of modification of 0·8 as determined spectrophotometrically (Fehske et al 1978). After the modification all α_1-AGP derivatives were purified by dialysis, lyophilized, and stored at 0 °C.

RESULTS

Circular dichroism measurements

α_1-AGP exhibits a typical intrinsic CD spectrum in the near u.v. range between 350 and 250 nm (Fig. 1) which was first reported and interpreted by Jirgensons (1976). The addition of one mole dipyridamole mole^{-1} α_1-AGP profoundly changes the CD spectrum observed (Fig. 1). As indicated by the difference spectrum, the Cotton effects observed in the presence of dipyridamole consist of two large negative bands, a smaller one around 330 nm and a larger one around 300 nm with two peaks at 300 nm and 310 nm respectively. The extrinsic Cotton effects depend on the dipyridamole concentration as shown for the two strong negative peaks at 300 and 310 nm, with a linear increase of the molar ellipticity at both wavelengths up to a molar drug to protein (D/P) ratio of one (Fig. 2). Increasing the dipyridamole concentration further has little effect on the intensity of both

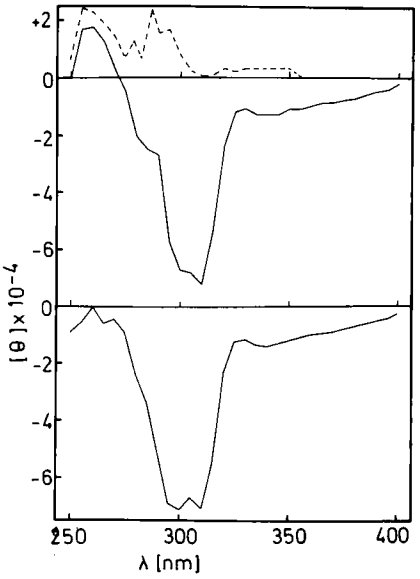

FIG. 1. Circular dichroism spectra of α_1-AGP alone and of α_1-AGP in the presence of one mole per mol^{-1} dipyridamole (upper part) and of the difference spectrum from subtracting the contributions of protein alone (lower part).

bands up to about five moles of dipyridamole mole^{-1} α_1-AGP (Fig. 2). A similar effect of the dipyridamole concentration was found for the negative extrinsic Cotton effects around 330 nm. Removal of sialic acid moieties from the α_1-AGP molecule according to Schmid et al (1973) decreased the intensity of the extrinsic Cotton effects of dipyridamole by about 25% as indicated for the negative band at 310 nm in Fig. 3 (closed and open circles). However, for

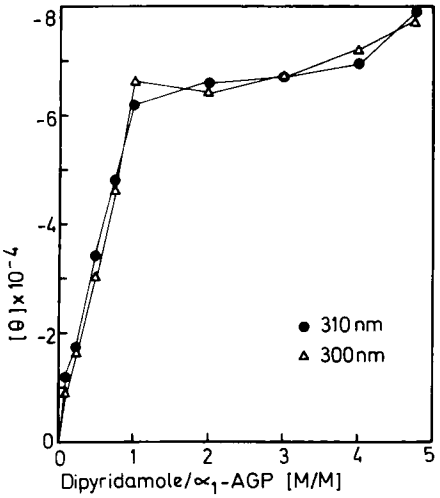

FIG. 2. The correlation between molar ellipticity ([θ]) and the molar dipyridamole/α_1-AGP ratio at the wavelength of the induced CD maxima.

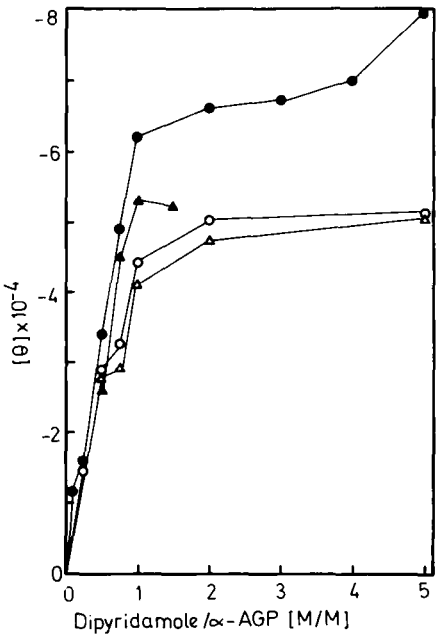

FIG. 3. The effect of protein desialysation on the correlation between molar ellipticity ([θ]) and the molar dipyridamole/α_1-AGP ratio. ●—● *native protein (310 nm)*; ▲—▲ dialysed and lyophilized protein (310 nm); △—△ desialysed protein (310 nm); ○—○ desialysed protein (300 nm).

removing the sialic acid moieties, the protein is heated in 0·1 M HCl for 1 h, then dialysed against water for 48 h, and then lyophilized. To investigate the influence of the second part of the procedure on the extrinsic Cotton effects another amount of the protein was only dialysed and lyophilized. For this protein derivative, approximately the same reduction of the extrinsic Cotton effects was found as for the desialized preparation (Fig. 3, closed triangles). Thus, the reduction of the extrinsic Cotton effects found for the desialized preparation does not indicate a specific role of the sialic acid moieties for the dipyridamole binding but are due to the isolation procedure. We did not observe a significant change of the correlation between the dipyridamole concentration and the intensity of the extrinsic Cotton effects for any of the protein preparations investigated in Fig. 3. Removal of the sialic acid moieties from the α_1-AGP molecule has only a small effect on the intrinsic Cotton effects of the protein alone.

Equilibrium dialysis measurements

Dipyridamole is strongly bound to α_1-AGP. By plotting the data obtained from the equilibrium dialysis measurements according to Scatchard (1949) a curved line was obtained (Fig. 4), indicating different sets of dipyridamole binding sites on the α_1-AGP molecule. Using the graphical method of Pennock (1973) the curved Scatchard plot could be resolved into two linear components indicating one high affinity binding site with an association constant as high as $15{\cdot}5 \times 10^6$ M^{-1} and a second site with much lower affinity (association constant $0{\cdot}4 \times 10^6$ M^{-1}) (Fig. 4).

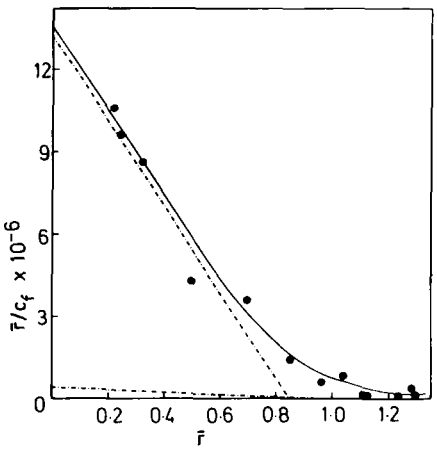

FIG. 4. Scatchard analysis of the binding of dipyridamole to α_1-AGP as determined by equilibrium dialysis. The dotted lines indicate the two sets of binding sites calculated by the method of Pennock (1973). The following data for n (number of sites) and k (association constant) were obtained: $n_1 = 0{\cdot}9$, $k_1 = 15{\cdot}5 \times 10^6$ (M^{-1}); $n_2 = 0{\cdot}9$, $k_2 = 0{\cdot}4 \times 10^6$ (M^{-1}).

U.v.-differences measurements

The presence of α_1-AGP characteristically changes the u.v. absorbance spectrum of dipyridamole as indicated by Fig. 5. A large positive band was found around 300 nm consisting of a shoulder at about 300 nm and a maximum at about 311 nm. A second positive but considerably smaller band can be seen around 444 nm (Fig. 5). In contrast to the association of the extrinsic Cotton effects of dipyridamole in the presence of α_1-AGP with only one mole of dipyridamole per mole of the protein the intensity of the changes of the u.v. absorbance increases linearly up to five moles of dipyridamole per mole α_1-AGP (Fig. 6). The relationship between the extrinsic Cotton effects of dipyridamole in the presence of α_1-AGP and of the changes of its u.v. absorbance at various D/P ratios is given in Fig. 7. It is obvious that the increase of the molar ellipticity at 310 nm is not directly correlated with the increase of ΔA at 310 nm.

Removing the sialic acid moieties from the α_1-AGP molecule according to the method described

above had no effect on the u.v. difference spectrum of dipyridamole in the presence of the protein.

A fairly similar u.v. difference spectrum to that found for dipyridamole in the presence of α_1-AGP (Fig. 5) can be observed for dipyridamole in the presence of human serum albumin. Also like the observation with α_1-AGP the u.v. difference spectrum in the presence of human serum albumin increases linearly up to a molar D/P ratio of five (Fig. 6). However, in contrast to the observation with α_1-AGP, no extrinsic Cotton effects could be found for dipyridamole in the presence of human serum albumin under similar conditions.

Effects of conformation perturbants

Like dipyridamole, progesterone has one high affinity binding site on the α_1-AGP molecular (Ganguly & Westphal 1968). Pronounced changes in the affinity of progesterone for this site have been reported due to the presence of several perturbants of the protein conformation. For example, high concentrations of NaCl, which stabilize the protein conformation, strongly increased the affinity of progesterone while agents like urea, which destabilize the protein conformation, decreased the affinity of progesterone (Ganguly & Westphal 1968). In contrast to these observations NaCl up to 4M has obviously no effect on the interaction of dipyridamole with α_1-AGP as indicated by the nearly unchanged extrinsic Cotton effects (Table 1) and by the unchanged u.v. difference absorbance (data not shown). However, high concentrations of urea decreased the intensity of both optical parameters of the interaction of dipyridamole with α_1-AGP (Table 1 and Fig. 8). The studies with urea further indicated that both

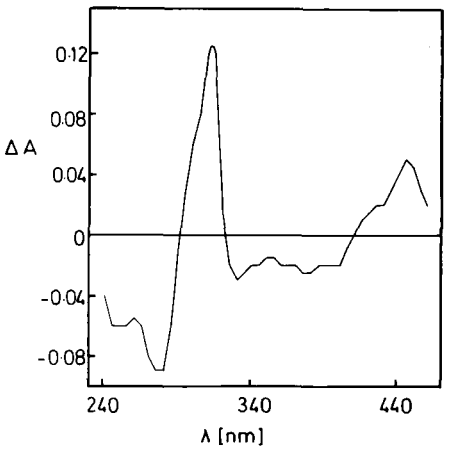

FIG. 5. Ultraviolet difference spectrum of dipyridamole in the presence of α_1-AGP at a molar ratio of one.

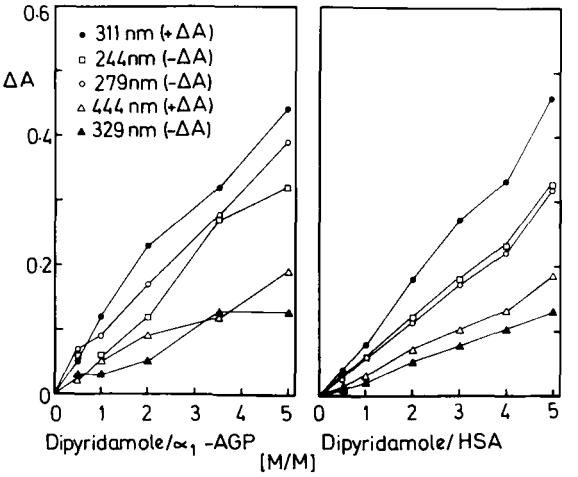

FIG. 6. The correlation of ultraviolet difference absorbance (ΔA) at several wavelengths and the molar dipyridamole/protein ratio for α_1-AGP (left) and human serum albumin (right).

parameters are not closely related since the extrinsic Cotton effects are clearly more sensitive to urea than the ΔA values (Table 1 and Fig. 8).

Like the observation on the binding of progesterone to α_1-AGP (Kerkay & Westphal 1969) low concentrations of mercury ions (4 mol mol^{-1} protein) have a distinct effect on the interaction of dipyridamole with the protein, as indicated by the decrease of the induced Cotton effects at 300 and 310 nm by about 25% (Table 1). The effect seems to depend directly on free mercury ions since it is reversible by EDTA (Table 1).

Effects of protein modification

The modification of about 0·8 tryptophan residues (data not shown) and about 1·2 tyrosine residues of

Table 1: effects of conformational perturbants on the extrinsic Cotton effects of dipyridamole in the presence of α_1-AGP. The data are given as molar ellipticity ([θ]) calculated with reference to the protein concentration at the wavelength of the two CD maxima at 300 and 310 nm.

		$[\theta] \times 10^4$	
		300 (nm)	310 (nm)
Control		6·6	6·2
NaCl	1 M	5·8	5·9
	2 M	5·4	5·7
	3 M	5·8	5·8
	4 M	6·2	6·1
Urea	3 M	5·2	5·3
	4 M	2·8	3·0
	6 M	1·4	1·6
	8 M	—	—
Hg^{++}	50 μM	4·5	5·0
Hg^{++}	50 μM + EDTA 25 mM	5·8	6·1

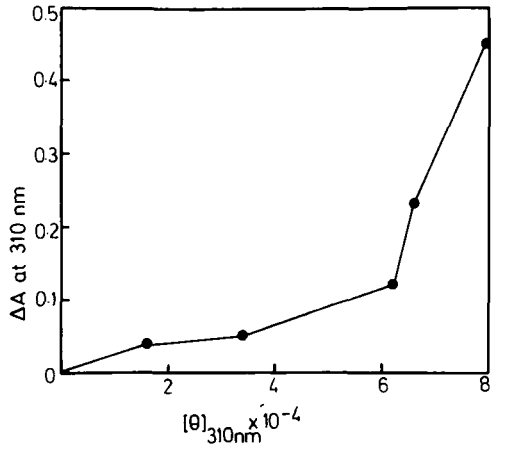

FIG. 7. The correlation between molar ellipticity ([θ]) and difference absorbance (ΔA) at 310 nm for various molar dipyridamole/α_1-AGP ratios (D/P).

α_1-AGP changes the extrinsic Cotton effects of dipyridamole bound to the protein only slightly (Fig. 9). However, increasing the number of tyrosine residues modified up to about 4·4 produces a distinct effect on the induced Cotton effects with about 30% reduction up to a dipyridamole/α_1-AGP ratio of four (Fig. 9). No significant alteration of the shape of the curves between molar ellipticity and molar dipyridamole/α_1-AGP ratio was observed for any of the modified proteins.

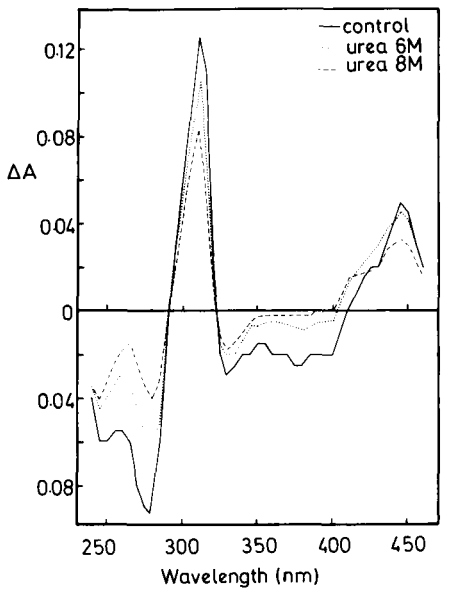

FIG. 8. Effect of urea on the ultraviolet difference spectrum of dipyridamole in the presence of α_1-AGP at a molar ratio of one.

DISCUSSION

Although the phenomenon of basic drug binding to α_1-AGP has been known for many years, the data available on the binding of drugs to pure α_1-AGP are limited (Piafsky & Knoppert 1978; Schley et al 1980; Brinkschulte & Breyer-Pfaff 1980). Moreover, no information is available about molecular aspects of basic drug binding to α_1-AGP. Since CD measurements have proved to be very helpful in evaluating qualitative aspects of drug interactions with serum albumins (Chignell 1970; Müller & Wollert 1975; Fehske et al 1979) we have investigated the interaction of several basic drugs with α_1-AGP using this method and have found with the vasoactive drug dipyridamole, that a specific and stable complex formation exists between it and α_1-AGP.

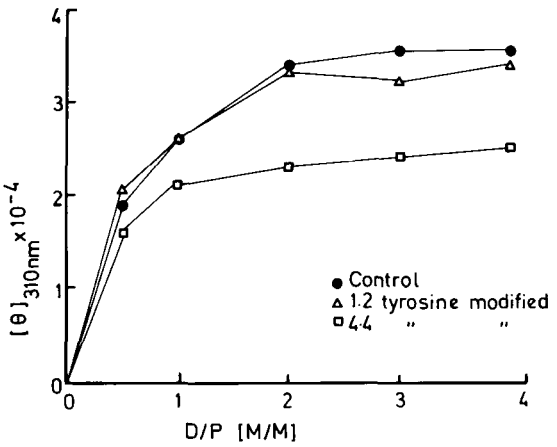

FIG 9. The effect of tyrosine residue modification on the correlation between molar ellipticity [θ] and the molar dipyridamole/α_1-AGP ratio. Control protein was treated similarly but without tetranitromethane. ● Control △ 1,2-tyrosine modified □ 4,4-tyrosine modified.

As indicated by the equilibrium dialysis experiments, dipyridamole binds via one high affinity and one low affinity site to α_1-AGP with a surprisingly high association constant for the first site which is higher than all other binding constants reported so far for drug binding to isolated α_1-AGP (Schley et al 1980; Brinkschulte & Breyer-Pfaff 1980) and is nearly as high as that of progesterone (Ganguly & Westphal 1968). Few drugs are known with similar or higher binding constants for human serum albumin. Only the association of dipyridamole with its high affinity binding site produces a typical and biphasic induced Cotton effect. The good correlation of the wavelength positions of the two induced CD bands at 300 and 310 nm with a shoulder at 300 nm and a maximum at 311 nm of the u.v. difference spectrum

of dipyridamole in the presence of α_1-AGP, clearly indicates that the observed Cotton effects are extrinsic in their origin and are originated by optical perturbations of electronic transitions in the ligand molecule (Chignell 1970). Thus, measuring changes of the induced Cotton effects of dipyridamole in the presence of α_1-AGP will specifically indicate changes of the complex formation only at the high affinity binding site. As far as we know, our findings about strong extrinsic Cotton effects in the case of the interaction of dipyridamole with α_1-AGP, whose intensities are in the same range usually found for the interaction of drugs with serum albumins, are the first observations that drug binding to this protein can exhibit the degree of specificity needed to induce optical asymmetry into the ligand molecule (Chignell 1970). Thus, a one point electrostatic attachment is not sufficient to explain the interaction of dipyridamole with the high affinity site.

In contrast, several pieces of evidence suggest that the high affinity site is located in a remote hydrophobic protein part of the glycoprotein: (1) The red shift of the u.v. absorbance that is generally observed for aromatic absorbance bands by changing the environment from polar (aqueous buffer) to a polar (organic solvent or hydrophobic area of the protein); (2) the findings that the sialic acid moieties, presumably a hydrophilic part of the molecule, are not involved in this site; (3) the observation that NaCl, which will shift the protein conformation to a more compact, ordered structure has almost no effect on the interaction with this site which is already a very compact area of the protein; (4) the observation that this site is sensitive to urea which will unfold the protein molecule to a random coil; (5) the findings that the modification of the highly lipophilic amino acid residues tyrosine and tryptophan has a distinct effect on the induced Cotton effects of dipyridamole bound to α_1-AGP. The location of this site and further amino acid residues involved are not yet known except that the sensitivity of this site for low concentrations of mercury may suggest that a disulphide bridge, rather than a free sulphydryl group (Kerkay & Westphal 1969), might be involved.

It might be that in the therapeutical situation not only the high affinity site may be important but also secondary sites, since α_1-AGP blood levels may range from 6–40 μM in healthy subjects (Piafsky & Borga 1977; Romach et al 1981) and the therapeutical plasma level of dipyridamole might be between 10 and 30 μM (Niewiarowski et al 1975). Unfortunately, the information we have about secondary sites is more limited. Equilibrium dialysis experiments revealed one secondary site but the linear increase of the ΔA values up to a molar D/P ratio of five may suggest more than one secondary site. Like the high affinity site, hydrophobic forces may play an important role in the binding to the secondary site(s) as indicated by the red shift of the u.v. absorbance and the sensitivity of the ΔA values to urea. In summary, the data reported have shown that α_1-AGP (orosumucoid) binds dipyridamole via a well-defined hydrophobic area of the protein part of the molecule with an extremely high affinity.

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